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# Phytochelatins Are Involved in Differential Arsenate Tolerance in *Holcus lanatus*<sup>1</sup>

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Arsenate tolerance is conferred by suppression of the high-affinity phosphate/arsenate uptake system, which greatly reduces arsenate influx in a number of higher plant species. Despite this suppressed uptake, arsenate-tolerant plants can still accumulate high levels of As over their lifetime, suggesting that constitutive detoxification mechanisms may be required. Phytochelatins are thiol-rich peptides, whose production is induced by a range of metals and metalloids including arsenate. This study provides evidence for the role of phytochelatins in the detoxification of arsenate in arsenate-tolerant *Holcus lanatus*. Elevated levels of phytochelatin were measured in plants with a range of tolerance to arsenate at equivalent levels of arsenate stress, measured as inhibition of root growth. The results suggest that arsenate tolerance in *H. lanatus* requires both adaptive suppression of the high-affinity phosphate uptake system and constitutive phytochelatin production.

Arsenate is an analog of phosphate, competing for the same uptake carriers in the root plasmalemma (Meharg and Macnair, 1992b). Arsenate tolerance has been identified in a number of plant species (Meharg, 1994; Sharples et al., 2000b). Tolerance in grasses results via suppression of the high-affinity phosphate/arsenate uptake system (Meharg and Macnair, 1992b). This suppression reduces arsenate influx to a level at which it is thought that the plant can detoxify it, presumably by constitutive mechanisms (Meharg, 1994). The theory is based on the fact that arsenate tolerance is achieved by a single gene, which codes for the suppressed phosphate/arsenate transport (Meharg et al., 1992; Meharg and Macnair, 1992b).

Despite this clear understanding of the processes controlling decreased arsenate uptake, tolerant grasses still assimilate As, albeit at much lower rates compared with non-tolerants. Nevertheless, assimilation over the life history of plants growing on contaminated soil can result in very high As concentrations, e.g. 3,470  $\mu\text{g g}^{-1}$  As in *Agrostis tenuis* and 560  $\mu\text{g g}^{-1}$  As in *Holcus lanatus* (Porter and Peterson, 1975). It is postulated that arsenate is transformed within plant cells to other less phytotoxic As species (Meharg, 1994). Metabolism to other As species has been observed in phytoplankton and macroalgae where arsenate is converted to arsenite, dimethylarsinic acid (DMA), and monomethylarsinic acid (MMA) (Phillips, 1990). These methylated forms of As are then metab-

olized to organophospholipids and arsenosugars (Phillips, 1990). Studies of terrestrial plants have only revealed the presence of arsenate and arsenite (Meharg, 1994; Van den Broeck et al., 1998), but a recent study on a range of terrestrial plants reported low concentrations of methylated As species, including MMA and DMA. However, the majority of the As was still present as the inorganic forms arsenate and arsenite, which are more phytotoxic (Koch et al., 2000).

Three studies recently have described the formation of As-phytochelatin complexes on exposure to arsenate, in arsenate-sensitive *Silene vulgaris* plants (Sneller et al., 1999), cell suspension cultures of *Rauvolfia serpentina* (Schmöger et al., 2000), and root cultures of *Rubia tinctorum* (Maitani et al., 1996). Phytochelatins (PCs) are thiol (SH)-rich peptides (common structure [ $\gamma$  - glu - cys]<sub>n</sub>gly in which  $n = 2-11$  [e.g. PC<sub>2</sub>, PC<sub>3</sub>, PC<sub>4</sub>]) whose production is induced by a range of heavy metals including Cd, As, Cu, and Zn (Grill et al., 1985). Previous studies of PC production in response to Cd and Cu have illustrated that although PCs are involved in the detoxification process, they are not responsible for metal tolerance (De Knecht et al., 1992; Schat and Kalff, 1992). However, the processes that govern arsenate tolerance have already been shown to be significantly different to those of other metals (Meharg, 1994). A role for PCs in the detoxification of arsenate was first suggested because of their induction by arsenate (Grill et al., 1987). This hypothesis was supported by evidence of the formation of As-SH complexes both in vivo and in vitro (Jocelyn, 1972; Scott et al., 1993). The gene encoding PC synthase (the enzyme responsible for the production of PCs from glutathione [GSH]) has recently been identified in *Arabidopsis* (Clemens et al., 1999; Ha et

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al., 1999; Vatamaniuk et al., 1999), *Triticum aestivum*, and *Schizosaccharomyces pombe* (Clemens et al., 1999; Ha et al., 1999). It has also been shown that a mutant *Arabidopsis* lacking the ability to synthesize PCs, was much more sensitive to arsenate than the wild type (Ha et al., 1999). Since then, the production of As-PC complexes has been unequivocally demonstrated, through purification of PCs by electrospray ionization mass spectroscopy (ESI-MS) (Schmöger et al., 2000) and x-ray absorption spectroscopy (Pickering et al., 2000). This study investigated whether As metabolism (methylation) or complexation by PCs was responsible for the enhanced ability of arsenate-tolerant plants to detoxify and accumulate arsenate.

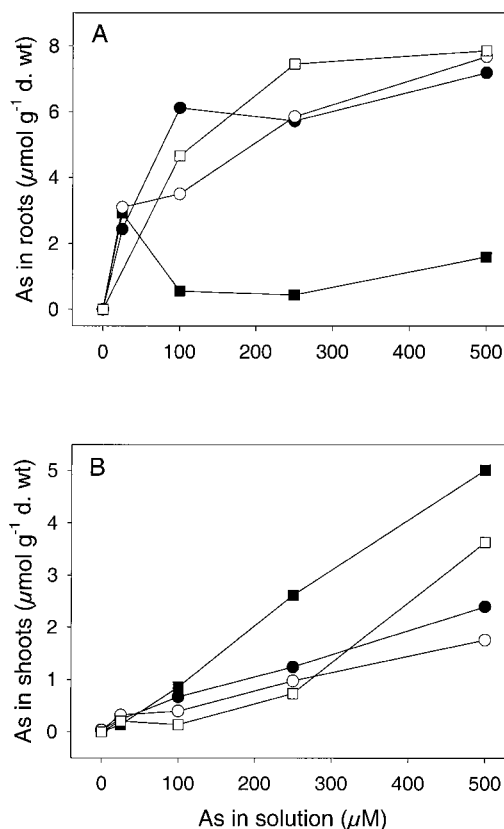
## RESULTS

### Effect of Increasing Arsenate Exposure on Root Growth and Arsenate Uptake

Inhibition of root growth was an accurate indicator of metal toxicity with large differences in As  $EC_{50}$  (effective concentration that inhibits root growth by 50%) between tolerant, intermediate, and non-tolerant clones (Table I). The most tolerant clone (M1) had an  $EC_{50}$  greater than 1,000  $\mu M$  As, whereas the  $EC_{50}$  of the least tolerant clone (NM2) was 3  $\mu M$  As (Table I). For three of the clones, root As concentrations were higher than in the shoots at all external concentrations (Fig. 1). The exception was non-tolerant clone (NM2) where root As remained low, yet shoot As continued to increase with increasing external arsenate (Fig. 1). NM2, the least tolerant clone, was expected to show elevated uptake, but because of the large concentration range used for this experiment, the growth of NM2 was completely inhibited above 25  $\mu M$  As. Therefore, As concentrations in roots and shoots of NM2 above this exposure level were measured on plants that were no longer growing.

### Assay of Acid-Soluble Thiols

Total PC production increased with external arsenate concentration for the two tolerant clones (M1 and M2) and the intermediate tolerant clone (NM1) (Fig. 2). The exception was the non-tolerant clone (NM2), where PC concentrations decreased above 25  $\mu M$  As



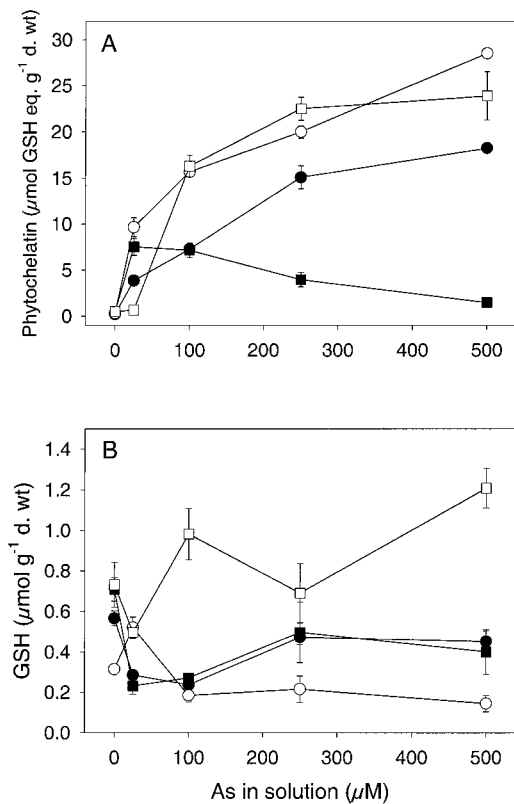
**Figure 1.** Concentration of As in the roots (A) and shoots (B) of four *H. lanatus* clones from an As contaminated (M) and an uncontaminated (NM) site. Plants were exposed to a range of arsenate concentrations for 7 d. Data represent the mean  $\pm$  SE ( $n = 3$ ). NM1, ●; NM2, ○; M1, ■; M2, □.

due to the death of the plants and corresponded with a decrease in As concentrations in NM2 roots (Fig. 1). Arsenate-tolerant (M1 and M2) plants produced higher concentrations of PC than the non-tolerant plant (NM2) at equal external concentrations, the intermediate tolerant plant showing intermediate PC production (Fig. 2). To gain an estimate of PC production at an equivalent level of stress in the four clones, curves were fitted to the total PC data (Sigmaplot 2000, SPSS, Chicago) to estimate the concentration of total PC at the arsenate  $EC_{50}$  concentrations (Table I). From this calculation, PC production was shown to be 15- to 20-fold higher in the tolerant clones (M1 and M2) than the non-tolerant clone (NM2) under equivalent stress (Table I). GSH concentrations were low compared with PC concentrations and there was no significant difference between GSH concentrations in the tolerant and non-tolerant clones (Fig. 2;  $P > 0.05$ ; data not shown). However, GSH concentrations in the two non-tolerant clones were very similar with initial depletion of GSH levels at low arsenate concentrations but some recovery at higher arsenate concentrations. This discrepancy could be due to the death of the plants above 25  $\mu M$  As, as normal cell functioning would be severely

**Table I.** Arsenate  $EC_{50}$  values and estimated PC production at  $EC_{50}$  concentration of *H. lanatus* clones from an uncontaminated (NM) and a contaminated (M) site

PC production was estimated from the dose response curves in Figure 2.

Code	As $EC_{50}$	Estimated PC
	$\mu M$	$\mu mol\ GSH\ equivalent\ g^{-1}\ dry\ wt$
NM1	40	4.5
NM2	3	1.5
M1	>1,000	28.5
M2	800	23.9



**Figure 2.** Concentrations of total PC (A) and GSH (B; equivalent  $\mu\text{mol GSH equivalent g}^{-1}$  dry weight) in four *H. lanatus* clones from an As contaminated (M) and an uncontaminated (NM) site. Plants were exposed to a range of arsenate concentrations for 7 d. Data represent the mean  $\pm$  SE ( $n = 3$ ). NM1,  $\bullet$ ; NM2,  $\blacksquare$ ; M1,  $\circ$ ; M2,  $\square$ .

disrupted before the plants died. GSH levels in the two tolerant clones were very different with depleted GSH levels in M1 and increased levels in M2.

#### As Analysis of $\text{EC}_{50}$ Samples

As concentrations in the roots were significantly ( $P < 0.05$ ) higher in the tolerant clone (M2) compared with the non-tolerant and intermediate tolerant clones at the  $\text{EC}_{50}$  exposure concentrations (Table II). In ad-

dition M3 had significantly higher root As concentrations than NM1 and NM3 but significantly lower root As than tolerant M2. This was not expected but may reflect the fact that this clone was from a contaminated site. Relative to the exposure concentrations, accumulation from the solution to the root was 10- to 60-fold lower in the tolerant clone (M2) compared with the remaining clones (NM1, NM3, and M3) (Table II). The arsenate exposure of the tolerant clone (M2) was 160 times higher than the non-tolerant clone (NM3), and yet the corresponding concentration in the roots was only three times higher in the tolerant (M2) compared with the non-tolerant clone (NM3) (Table II).

#### As Speciation by Liquid Chromatography-Mass Spectrometry

No MMA, DMA, or tetramethylarsonium ion (TMA) were detected in root or shoot samples of *H. lanatus* from the As-Cu contaminated site using liquid chromatography-mass spectrometry (LC-MS) ESI.

#### Identification of Acid-Soluble Thiols by HPLC

Total PC concentrations were significantly higher ( $P < 0.001$ ) in the tolerant clone (M2) than in the remaining three clones at the  $\text{EC}_{50}$  concentrations (Fig. 3, Table II). This might imply that tolerant plants have a greater capacity for PC production than non-tolerant plants at equivalent levels of stress. The ratio of PC-SH/As was calculated to determine the number of SH groups available for coordination of As. PC concentrations were calculated in  $\mu\text{mol GSH equivalent}$ , this therefore accounts for the fact that different PC species,  $\text{PC}_2$ ,  $\text{PC}_3$ , and  $\text{PC}_4$  contain 2, 3, and 4 SH groups, respectively. The ratio of PC-SH/As differed with plant tolerance (Table II). The most tolerant clone (M2) had the highest PC-SH/As ratio of 1.77, whereas the non-tolerant clone (NM3) had the lowest PC-SH/As ratio of 0.25. Following this trend it was expected that of the two intermediate clones, NM1 would have a higher total PC content than M3 due to its higher As  $\text{EC}_{50}$  value. However, this was

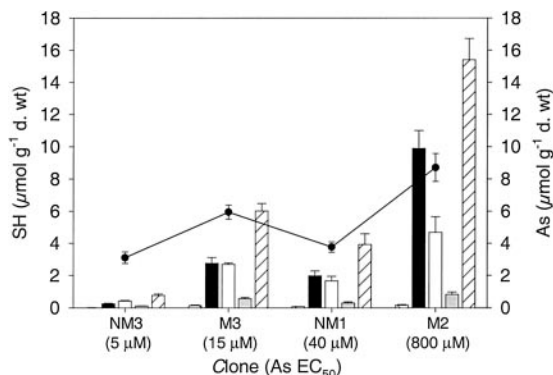
**Table II.** As, total PC, and GSH concentrations in roots of *H. lanatus* clones of varying sensitivity exposed to their own As  $\text{EC}_{50}$  concentration

As accumulation from solution to root (As content of control root samples was  $< 0.003 \mu\text{mol g}^{-1}$  dry wt). Data represent the mean  $\pm$  SE ( $n = 4$ ).

Clone	As $\text{EC}_{50}$ ( $\mu\text{M}$ )	As Root Conc	As Accumulation (Root/Solution)	Total PC	GSH	Ratio PC-SH/As	Ratio $\text{PC}_2$ : $\text{PC}_3$ : $\text{PC}_4$
		$\mu\text{mol g}^{-1}$ dry wt		$\mu\text{mol GSH}$ equivalent $\text{g}^{-1}$ dry wt	$\mu\text{mol g}^{-1}$ dry wt		
NM1	40	$3.8 \pm 0.3^a$	0.10	$3.9 \pm 0.7^b$	$0.08 \pm 0.01^a$	1.04	7:6:1
NM3	5	$3.1 \pm 0.4^a$	0.62	$0.8 \pm 0.1^a$	$0.01 \pm 0.001^b$	0.25	2:4:1
M2	800	$8.7 \pm 0.9^b$	0.01	$15.4 \pm 1.3^c$	$0.16 \pm 0.04^a$	1.77	12:6:1
M3	15	$5.9 \pm 0.4^c$	0.40	$6.0 \pm 0.5^b$	$0.14 \pm 0.03^a$	101	5:5:1

<sup>a,b,c</sup> Values followed by the same letter do not differ at  $P < 0.05$ .





**Figure 3.** Concentrations of arsenate induced PCs (PC<sub>2</sub>, PC<sub>3</sub>, PC<sub>4</sub>), total PC, and GSH ( $\mu\text{mol g}^{-1}$  dry weight) in roots of four *H. lanatus* clones exposed to their own arsenate EC<sub>50</sub> concentration for 7 d. Data represent the mean  $\pm$  SE ( $n = 4$ ). GSH (cross hashed), PC<sub>2</sub> (■), PC<sub>3</sub> (□), PC<sub>4</sub> (▨), total PC (left hashed), As root concentration (●).

not the case, as the total PC value corresponded with As root concentrations, not EC<sub>50</sub> values, resulting in both clones having a PC-SH/As ratio of 1.0 (Table II). GSH concentrations were higher in the arsenate-treated plants than the control plants for all four clones (data not shown). Furthermore, the non-tolerant clone (NM3) had a significantly lower GSH concentration than the remaining clones, although overall GSH concentrations were low in relation to PC levels ( $P = 0.001$ ; Table II).

The production of different chain length PCs varied between the four clones (Fig. 3). In the non-tolerant clone (NM3), PC<sub>3</sub> was the dominant form, although overall total PC-SH concentrations were low (Fig. 3, Table II). In the intermediate clones (NM1, M3), PC<sub>2</sub> and PC<sub>3</sub> concentrations were approximately equal as indicated by the PC ratios, however, in the arsenate-tolerant clone (M2) PC<sub>2</sub> was dominant (Fig. 3, Table II). PC<sub>4</sub> remained at low concentrations in all four clones exposed to arsenate.

## DISCUSSION

Over a range of arsenate concentrations, tolerant *H. lanatus* clones produced higher concentrations of PC than non-tolerant clones at equal external concentrations. When these data were modeled it was revealed that at the arsenate EC<sub>50</sub> concentrations, estimated PC production was 15- to 20-fold higher in tolerant compared with non-tolerant clones. A second set of clones subsequently were exposed to their own arsenate EC<sub>50</sub> concentrations, and the results were confirmed with a tolerant *H. lanatus* clone producing significantly higher PC concentrations than non-tolerant and intermediate tolerant clones. These results were in contrast to previous findings where PC production at Cd and Cu EC<sub>50</sub> concentrations was equal at equivalent levels of stress (De Knecht et al., 1992; Schat and Kalff, 1992).

A difference in the distribution of PCs over chain length classes, in tolerant and non-tolerant plants, was also observed in response to arsenate. Previous studies of arsenate induced PCs have reported PC<sub>2</sub> as the dominant species over a range of arsenate concentrations (Sneller et al., 1999; Schmöger et al., 2000). In this study PC<sub>2</sub> was dominant in the non-tolerant clone, whereas PC<sub>3</sub> was dominant in the most tolerant clone. The speciation in arsenate-tolerant and non-tolerant plants has not been examined previously, however, in studies of Cd and Zn tolerance, no difference in the PC<sub>2</sub>:PC<sub>3</sub>:PC<sub>4</sub> ratio was reported between tolerant and non-tolerant plants (De Knecht et al., 1992; Harmens et al., 1993).

The variation in tolerance of the clones investigated is very clear with a 300-fold difference in EC<sub>50</sub> values. This significant difference in tolerance is due to suppression of the high-affinity phosphate/arsenate uptake system, reducing arsenate influx to a much lower rate and this has been shown conclusively in *H. lanatus* clones from the populations studied here (Meharg and Macnair, 1992b). However, arsenate-tolerant plants do assimilate arsenate to higher concentrations than non-tolerant plants over their life-history, indicating that they must also have a successful internal detoxification mechanism.

The findings presented here indicate a different role for PCs in arsenate tolerance compared with Cd and Cu tolerance. For Cd and Cu, PCs provide a constitutive detoxification mechanism that is the same in tolerant and non-tolerant plants (De Knecht et al., 1992; Schat and Kalff, 1992). However, elevated PC production in arsenate-tolerant plants might imply that this response is adaptive, providing an additional mechanism of arsenate tolerance along-side suppression of arsenate uptake. This could be explained by inherently increased PC synthesis capacity (Vatamaniuk et al., 1999), but there is strong evidence against this conclusion. First, the adaptation of suppressed arsenate uptake is under single gene control (Meharg et al., 1992). Suppressed influx was shown to cosegregate with tolerance, demonstrating that the gene coding for tolerance also coded for suppression of the arsenate uptake system (Meharg and Macnair, 1992a). If elevated PC production was an adaptive response, this would imply that this same gene for suppression also coded for differential PC production, or would at least suggest that these genes were linked, this is unlikely. However, as there is a degree of quantitative variation among tolerant plants, it is possible that the gene for PC synthase is acting as a hypostatic modifying factor to the tolerance allele (Macnair et al., 1992). Second, arsenate-tolerant plants have reduced rates of influx compared with non-tolerant plants (Meharg and Macnair, 1992b), and as PC responses have been shown to be related to the level of tolerance, they are also likely to be related to arsenate influx. Rates of influx were not measured in this study, however, a large number of clones from the same *H. lanatus* populations have

been characterized for their arsenate tolerance and influx rates by Meharg and coworkers (Meharg and Macnair, 1992a, 1992b). Therefore, conclusions made in this study are based on previous knowledge of the influx rates of arsenate-tolerant and non-tolerant *H. lanatus*.

In these experiments, As and PC concentrations were measured after 7 d. The rapid rate of arsenate influx in non-tolerant plants initially would result in a high toxic burden in the cells of these plants. PCs would be induced, but the toxicity of arsenate could lead to disruption of the transport process and also disruption of PC production. Furthermore, arsenate/phosphate transport is under feedback regulation by phosphate and potentially by arsenate in non-tolerant plants (Meharg and Macnair, 1992b; Sharples et al., 2000a). This regulation could alter arsenate assimilation rates over the 7-d experiment. In contrast, arsenate influx into tolerant plants would be significantly lower, resulting in lower and more constant toxic burdens, thus allowing arsenate transport and PC production to continue unaffected.

An additional factor that might explain the differential PC production in response to arsenate and Cd and Cu is the different stability of the As-PC complex. The stability of As-PC complexes is different from that of Cd or Cu-PC complexes at vacuolar pH. There are two lines of evidence for this. First, As-PC complexes destabilize in alkaline buffer but not in weakly acidic buffer (Schmöger et al., 2000). Second, a comparison of derivitization of arsenate induced PCs using (a) post-column DTNB derivitization at acid pH and (b) pre-column mBBR derivitization at pH 8.2 (Sneller et al., 2000) showed that As-PC complexes only dissociated under alkaline conditions. Cd-PC complexes are transported across the tonoplast into root vacuoles where they eventually dissociate due to the acidic vacuolar pH (Vogeli-Lange and Wagner, 1990; Salt and Rauser, 1995; Johanning and Strasdeit, 1998). From there, it is suggested that they either degrade or are shuttled back into the cytoplasm. If As-PC complexes are also transported into the root vacuole, then under the acidic conditions present, they might remain stable preventing re-oxidation of As (III) and allowing accumulation of high concentrations of As-PC complexes in arsenate-tolerant plants. In non-tolerant plants this would also be possible, however, as described above, the differential influx and feedback regulation would prevent the formation of high levels of As-PC complexes due to the toxic effect of arsenate in non-tolerant plants.

This differential stability of As-PC complexes could also affect the production of longer chain PCs (Hayashi et al., 1991) and might explain why PC<sub>2</sub> is the dominant form in the arsenate-tolerant clone. In PC biosynthesis, shorter chain PCs act as substrates for longer chain PCs (Hayashi et al., 1991). The strong binding of As to PC<sub>2</sub> could, therefore, result in a shortage of substrate for the production of PC<sub>3</sub>. Sneller

et al. (1999) reported that in arsenate-sensitive *S. vulgaris* PC<sub>3</sub> and PC<sub>4</sub> only started to form 24 to 48 h after exposure. This is in contrast to studies of Cd exposure where PC<sub>3</sub> concentrations exceeded PC<sub>2</sub> concentrations after only 2 h (Grill et al., 1987). The ratio of PC-SH/As also differed with plant tolerance (ranging from 0.25–1.77) with the tolerant clone (M2) having the highest PC-SH/As ratio. In a study of reconstituted As-PC complexes, three SH groups from two PC<sub>2</sub> molecules were shown to coordinate one As ion, leaving one SH un-coordinated (Schmöger et al., 2000), whereas in arsenate-sensitive plants the ratio of PC-SH/As was  $4 \pm 1.5$  (Sneller et al., 1999). The results reported here indicate that not all the As is being complexed by PC in the *H. lanatus* clones with least complexation in the non-tolerant clones.

This study has shown that *H. lanatus* is not converting arsenate or arsenite into organic As species. Other studies of As speciation, in a wide range of terrestrial plants and vegetables, reported that As was present either totally or predominantly as arsenate and arsenite (Helgesen and Larsen, 1988; Van den Broeck et al., 1998; Koch et al., 2000). The detection of free arsenate and arsenite species does not preclude the presence of PC in these plants as the HPLC-ICP MS techniques used in speciation did not set out to look for PC complexes, and as noted above, these complexes are constitutive under a range of conditions. However, this finding is important as inorganic As species are more phytotoxic than organic species (Cullen and Reimer, 1989). The lack of evidence for As methylation and the incomplete complexation of As in *H. lanatus* by PCs suggests that compartmentalization of As in a non-PC coordinated form may also be occurring in terrestrial plants.

So what is the role of PC complexation in arsenate tolerance? Arsenate tolerance is a result of suppression of the high-affinity phosphate uptake system, and this has been demonstrated in a range of terrestrial higher plants (Meharg, 1994). However, tolerant plants are still capable of enhanced accumulation and storage of arsenate compared with non-tolerant plants. Evidence presented here indicates that the adaptation of grasses to withstand high levels of arsenate relies on constitutive production of As-PC complexes. Furthermore, elevated levels of PC in arsenate-tolerant plants are due to differential influx in tolerant and non-tolerant plants resulting in differential toxicity.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Seed of *Holcus lanatus* was collected from an uncontaminated site (University of Exeter, Devon; code NM) and an As-Cu contaminated site (Gawton United mine, Devon Grid ref. SX452688; code M). Clonal plants were grown from seed of each population, and plants were maintained in a glasshouse and grown in John Innes compost No. 2.

Tillers of clonal plants from both populations were screened for arsenate tolerance using a standard rooting test over 7 d (Wilkins, 1957). Clones with a range of tolerance to arsenate (non-tolerant, tolerant, and intermediate tolerant) were selected from each population where possible, as measured by inhibition of root-length. Detailed dose-response experiments over 7 d were then conducted to determine their  $EC_{50}$  (effective concentration that inhibits root length by 50%).

### Dose Response Exposure Experiment

Two clones were selected from each of the populations for the dose-response experiment based on their  $EC_{50}$  values (Table I). NM1 and NM2 from the uncontaminated site were intermediate tolerant and non-tolerant, respectively, whereas M1 and M2, from the contaminated site, were arsenate tolerant. Un-rooted tillers of these *H. lanatus* clones were placed in a phosphate free nutrient solution containing 0.2 mM  $Ca(NO_3)_2$  + 0.2 mM  $KNO_3$  + 0.1 mM  $MgSO_4$  (pH 5.5, adjusted with HCl). Tillers were then grown for 10 d prior to arsenate exposure in a growth room with a 16-h photoperiod, and a day/night temperature of 21°C/17°C. Rooted tillers were exposed to a range of arsenate concentrations and grown for 7 d under the conditions described above. Treatments were: basal nutrient solution amended with 0, 10, 50, 100, 500, and 1,000  $\mu M$  As added as  $Na_2HAsO_4$  (pH 5.5, adjusted with HCl). For each treatment, 12 tillers were placed in three 180-mL polystyrene cups containing 170 mL of control or arsenate-amended solution with three replicate groups of pots per treatment. Longest root-length was measured at the beginning and end of the exposure period. After 7 d all plant material was rinsed in deionized water and blotted. From each replicate group, root material was separated into two sub-samples. One subsample was frozen in liquid nitrogen, freeze-dried, and stored under vacuum at 20°C for analysis of glutathione (GSH) and PC complexes. The second subsample was rinsed in ice-cold phosphate buffer and blotted (1 mM  $Na_2HPO_4$  + 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid] + 0.5 mM  $Ca(NO_3)_2$ ) to ensure desorption of arsenate from the roots free space (Asher and Reay, 1979). This subsample and all shoot material were oven dried at 70°C for 48 h and analyzed for As as described later.

### $EC_{50}$ Exposure Experiment

A second set of clones were chosen for the  $EC_{50}$  experiment based on their arsenate  $EC_{50}$  values (Table II). The clones used were different from those used in the dose-response experiment due to repeated problems with leaf rust. NM2 was replaced with NM3 a clone with very similar  $EC_{50}$ , M1 (tolerant) was replaced with M3 (intermediate) so we could compare the PC production in intermediate-tolerant clones from a contaminated and uncontaminated site. NM1 and NM3 from the uncontaminated site were intermediate tolerant and non-tolerant, respectively. M2 was a tolerant clone from the contaminated site, whereas M3 was an intermediate tolerant clone from the contaminated site.

Tillers of these clones were rooted in basal nutrient solution for 10 d as described above. They were then exposed to 0  $\mu M$  arsenate or their own arsenate  $EC_{50}$  concentration (Table II) and grown for 7 d. Longest root-length was measured at the beginning and end of the exposure period. For each treatment, 20 tillers were placed in five 180-mL polystyrene cups containing 170 mL of control or arsenate-amended solution with four replicate groups of pots per treatment. After 7 d all plant material was rinsed in ice-cold phosphate buffer (see above) and blotted. Root material within each replicate group was frozen in liquid nitrogen, freeze-dried, and stored (see above) for analysis of As, GSH, and arsenate-induced PCs.

### Extraction and Assay of Acid-Soluble Thiols

Total acid soluble thiols (TAST) in the dose-response samples were assayed according to De Vos et al. (1992). Extraction was carried out by grinding 10 to 20 mg of freeze-dried root material (using a mortar, pestle, and quartz sand) in 2 mL 5% (w/v) sulfosalicylic acid with 6.3 mM diethylenetriaminepentaacetic acid (DTPA) (pH < 1) at 0°C. After centrifugation at 10,000g for 15 min (4°C) the supernatants were immediately assayed. The concentration of TAST was determined using Ellman's reagent (Ellman, 1959). Supernatant (300  $\mu L$ ) was mixed with 630  $\mu L$  of 0.5 M  $K_2HPO_4$  and the absorbance measured after 2 min at 412 nm (30°C). After addition of 25  $\mu L$  of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution (10 mM DTNB, 0.143 M  $K_2HPO_4$ , 6.3 mM DTPA, pH 7.5) the absorbance was re-measured after 2 min. The increase in absorbance was corrected for the absorbance of DTNB. Values were calculated using the molecular extinction coefficient  $\lambda_{412} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$  (Harmens et al., 1993). Total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) were determined by the GSSG recycling method (Anderson, 1985) using GSSG as a standard. The concentration of PC was calculated as:  $PC = TAST - (TG - GSSG)$ . The recovery and oxidation of acid-soluble thiols were determined using GSSG as an external and internal standard.

### Identification of Acid-Soluble Thiols by HPLC

Root material from the  $EC_{50}$  experiment was used for HPLC analysis of GSH and arsenate-induced PCs. GSH and different chain-length PCs ( $PC_2$ ,  $PC_3$ ,  $PC_4$ ) were separated out using this method. Freeze-dried root material (20 mg) was ground (using a mortar, pestle, and quartz sand) in 2 mL of 6.3 mM DTPA with 0.1% (v/v) trifluoroacetic acid at 0°C. *N*-acetyl Cys was added during grinding as an internal standard. After centrifugation at 10,000g for 15 min (4°C) the supernatant was filtered through a Costar Spin-X centrifuge tube with a nylon filter (0.22  $\mu m$ ). The derivitization procedure was that of Sneller et al. (2000): 450  $\mu L$  of 200 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid buffer pH 8.2, containing 6.3 mM DTPA was mixed with 10  $\mu L$  of 25 mM monobromobimane. To this mixture, 250  $\mu L$  of the filtered supernatant was added, and derivitization was carried out for 30 min at 45°C in a covered water bath.



The reaction was stopped by the addition of 300  $\mu\text{L}$  of 1 M methansulphonic acid. Samples were stored in the dark at 4°C until HPLC analysis.

GSH and arsenate-induced PCs were separated on a Nova-Pak  $\text{C}_{18}$  column (6 nm, 4  $\mu\text{m}$ , 3.9  $\times$  300 mm, catalogue no. 11695, Waters, Milford, MA) at 37°C. Before injection the column was equilibrated in 12% (v/v) methanol and 88% (v/v) water both containing 0.1% (v/v) trifluoroacetic acid. The sample was injected and eluted with a slightly concave gradient of 12% to 25% (v/v) methanol for 15 min and then a linear gradient from 25% to 50% (v/v) methanol (v/v) from 15 to 50 min. Fluorescence was monitored using a Waters 474 fluorescence detector. The volume of derivitized sample injected was 6 to 25  $\mu\text{L}$  depending on the concentration of PC. Reduced glutathione (GSH) was used as an external standard. PC concentrations were calculated as  $\mu\text{mol}$  GSH equivalent  $\text{g}^{-1}$  dry weight and corrected for derivitization efficiency (Sneller et al., 2000).

### Total As Analysis

Total As was extracted by digesting 100 to 300 mg dry root or shoot material in 5 mL of Aqua Regia (1:4, v/v,  $\text{HCl}:\text{HNO}_3$ ) at reflux temperature for 2 h, after which the sample was filtered and the volume adjusted to 25 mL with 12.5% (v/v)  $\text{HNO}_3$ . Total As concentrations were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES, Jobin-Yvon JY38+).

### Mass Spectroscopy Analysis

Samples of *H. lanatus* roots and shoots collected from the As-Cu contaminated mine site were analyzed for the following methylated As species: MMA, DMA, and TMA. Shoot samples were freeze-fractured in liquid nitrogen and extracted using methanol and water (1:1, v/v). Analysis by LC-MS with ESI was conducted on methanol extracts (model HP1100 series LC-MS ESI, Hewlett-Packard, Bracknell, UK). Chromatographic and mass spectrometer conditions were those outlined by Inoue et al. (1999). An Altech (UK) SCX 5-m, 250-mm-length, 4.6-mm-i.d. cation exchange column was chosen to conduct separation with a mobile phase of  $\text{HNO}_3$  (8 mM)/ $\text{NH}_4\text{NO}_3$  (5 mM). The column was maintained at a temperature of 30°C and a flow rate of 0.4  $\text{mL min}^{-1}$ . Injection volume was 25  $\mu\text{L}$ . The MSD was run in positive mode with 5.0-V ion energy. The quadrupole temperature was 99°C and the gas temperature 300°C with a drying gas flow rate of 10  $\text{L min}^{-1}$  and a nebulization pressure of 45 pounds per square inch. Retention times and mass-spectral fingerprints of MMA, DMA, and TMA standards were used to identify these compounds in plant tissues.

### Statistical Analysis

Total As, PC, and GSH concentration data were analyzed using one-way analysis of variance. Means were compared using Tukeys multiple comparison test (Minitab v. 13.1, Minitab, State College, PA).

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